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Inoculation Techniques Used to Quantify Aflatoxin Resistance in Corn

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ABSTRACT

The development of *Aspergillus flavus* inoculation techniques has played an important part in developing corn (*Zea mays* L.) germplasm resistant to aflatoxin contamination. Corn genotypes evaluated for aflatoxin resistance in field studies must be artificially inoculated due to the sporadic nature of aflatoxin contamination from year to year. A number of different inoculation techniques are used by researchers in the South and Midwest. Field inoculation techniques either wound

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developing kernels or leave the kernels intact. Non-wounding techniques apply *A. flavus* conidia to exposed silks or silks inside the husks without damaging kernels. Wounding techniques deliver *A. flavus* conidia onto kernels that have been mechanically damaged. Inoculation techniques utilizing ear feeding insects to vector conidia have also been used in field studies. Environmental conditions such as ambient temperature and drought stress appear to have a significant impact on artificial inoculations. Laboratory evaluation techniques have been developed to confirm aflatoxin resistance identified in corn genotypes in the field. Color mutants and transformants of *Aspergillus* spp. have been used in field and laboratory studies to identify resistant genotypes. More efficient, less labor intensive, and less costly inoculation techniques need to be developed to aid in the production of aflatoxin resistant corn hybrids.

INTRODUCTION

Field studies in 1971 and 1972 established aflatoxin contamination as a preharvest problem in corn (Anderson et al., 1975). These studies initiated the evaluation of corn genotypes for sources of resistance to *Aspergillus flavus* kernel infection and aflatoxin contamination (Lillehoj et al., 1976; Widstrom et al., 1981). Since aflatoxin contamination is sporadic from growing season to growing season, inoculation techniques were developed to uniformly infect corn ears with *A. flavus* (King and Scott, 1982; Tucker et al., 1986). The development of inoculation techniques is difficult because *A. flavus* is a weak pathogen. Also, environmental conditions have a significant impact on kernel infection and aflatoxin accumulation (Widstrom et al., 1990). The inoculation techniques first developed could not identify corn genotypes that were resistant to *A. flavus* with any consistency. Thus, progress in developing resistant germplasm was slowed. Significant progress has been made in the last 15 years in developing inoculation techniques that can be used in the field or laboratory to identify corn genotypes with resistance to *A. flavus* and aflatoxin contamination.

A number of reports are available on a myriad of techniques used to artificially inoculate corn ears with *A. flavus* (Kang and Moreno, 2002; King and Wallin, 1983; Moreno and Kang, 1999; Scott and Zummo, 1987; Wilson et al., 1989). This report discusses inoculation techniques that are currently being used to evaluate corn genotypes for resistance to *A. flavus* and aflatoxin in corn in the southern and midwestern United States. Inoculum production and factors that have an impact on field evaluations will also be discussed.

EVALUATION OF AFLATOXIN RESISTANCE IN THE FIELD

Inoculation techniques used in field evaluations can be classified as wounding or non-wounding types. Non-wounding techniques mimic natural infection and allows the identification of corn germplasm that may have resistance mechanisms found on kernel surfaces such as waxes or thickened pericarps. Wounding techniques are generally more consistent in producing kernel infection and subsequent aflatoxin production. However, since kernels are damaged during inoculations, only genotypes with internal mechanisms of resistance can be identified.

Non-wounding Inoculation Techniques

One of the more commonly used non-wounding inoculation techniques in field studies involves spraying or atomizing small volumes of *A. flavus* conidia suspensions on silks exposed from the husks of ears (Jones et al., 1980; Payne et al., 1988). In a North Carolina study, silks were spray inoculated when they had turned yellow-brown in an effort to monitor aflatoxin contamination during ear development (Payne et al., 1988). After inoculation, ears were covered with a plastic bag for 3 days. Spray inoculations were used in studies in West Africa to identify aflatoxin resistant genotypes (Cardwell et al., 2000). Cycles of selection of four maize genotypes were inoculated by atomizing conidia on the silks and covering the ears with pollination bags. A spray technique has been developed that can be used to inoculate large field tests (Windham and Williams, 1999). Hybrids were evaluated for resistance to aflatoxin using a Solo backpack sprayer (Solo, Newport News, VA). A spore suspension containing 9×10^7 conidia/ml was sprayed on silks and husks when silks began emerging and spray treatments continued weekly for 5 weeks. The mean level of aflatoxin contamination for 45 commercial hybrids inoculated with this method was 2,208 ppb. Hybrids inoculated with the backpack sprayer had aflatoxin contamination at levels similar to hybrids inoculated with a wounding inoculation technique. Spray inoculation techniques have also been used to study the interactions of southwestern corn borer (*Diatraea grandiosella* Dyar) and *A. flavus*. Conidia were sprayed on silks using an Idico tree-marking gun (Idico Products Co., New York, NY) fitted with a spray nozzle (Windham et al., 1999). High levels of aflatoxin were found in ears inoculated with *A. flavus* and infested with southwestern corn borer.

Another non-wounding inoculation technique is the silk channel technique (Zummo and Scott, 1989). An Idico tree-marking gun fitted with

a 14-gauge needle was used to inject *A. flavus* conidia into the silk channel 6 days after midsilk (50% of the plants in a plot had silks emerged). Kernel infection of inbreds inoculated with the silk channel technique was similar in inbreds inoculated using wounding inoculation techniques.

Applications of granular material infected with *A. flavus* has been used to inoculate corn ears in field studies (Olanya et al., 1997). Corn seed infected with *A. flavus* and spread within plots to inoculate developing ears has been successfully used in field evaluations in the Coastal Bend of Texas (G. Odvody, pers. comm). Various formulations of alginate pellets containing *A. flavus* mycelia were evaluated for seeding agricultural fields (Daigle and Cotty, 1995). Atoxigenic strains of *A. flavus* cultured on long-grain rice were applied to fields to control aflatoxin in corn and peanut (Bock and Cotty, 1999; Cotty, 1994). Seed infected with an atoxigenic strain of *A. flavus* has been successfully used to control aflatoxin contamination in cottonseed in the southwestern United States (Dorner et al., 1998, 1999). Preliminary studies are being conducted by the authors to adapt this technology to inoculate corn in field studies with toxigenic strains of *A. flavus*. This technique is less labor intensive than other inoculation techniques and provides a more natural method of infection of the developing corn ears.

Wounding Inoculation Techniques

Wounding inoculation techniques commonly used to evaluate corn genotypes include the knife, pinbar, pinboard, side-needle, toothpick, and punch drill/pipe cleaner techniques. The knife technique has been the inoculation method of choice for evaluating corn genotypes for resistance in south Georgia (Widstrom et al., 1981, 1982, 1986, 1996). This technique involves dipping the tip of a grafting or paring knife into a spore suspension and inserting the blade through the husk and into the mid-section of an ear ca. 20 days after silking (Widstrom et al., 1981, 1982). In one study, ears inoculated with the knife technique had higher levels of aflatoxin contamination than ears inoculated with a needle or a multiple puncture technique (Widstrom et al., 1990). Tropical hybrids inoculated with the knife technique had lower levels of aflatoxin contamination than adapted hybrids in field evaluations (Widstrom et al., 1996). In North Carolina, ears of a commercial hybrid inoculated with the knife technique had aflatoxin levels during ear development comparable to ears that were inoculated by spraying spores on silks (Payne et al., 1988).

The pinbar technique utilizes a single 100-mm-long row of 35 sewing needles mounted in a plastic bar with ca. 6 mm of the points exposed (King and Scott, 1982). The needles are dipped in to an *A. flavus* spore suspension

(2×10^7 conidia/ml), aligned parallel with ear axis, and pressed through the husk and into kernels. Resistance to *A. flavus* can be quantified by determining kernel infection of kernels in rows adjacent to the wounded kernels (King and Scott, 1982) or by bulking wounded kernels with non-wounded kernels for aflatoxin analyses (Campbell and White, 1994; Tubajika and Damann, 2001; Tubajika et al., 2000). This technique is used by researchers in Louisiana for field evaluations. Environmental conditions favorable to disease development may result in extremely high aflatoxin contamination levels that limit the ability to separate resistant from susceptible genotypes.

The pinboard technique was developed to evaluate corn genotypes for resistance in the midwestern United States (Campbell and White, 1994). The Midwest has an environment which is often not conducive to *A. flavus* development and subsequent aflatoxin contamination. The pinboard inoculator was developed at the University of Illinois and consists of a pinboard of seven rows of 23 steel pins. The pinboard is attached to a spray gun which is connected to a Solo backpack sprayer. To inoculate a developing corn ear, the pinboard is aligned with the ear axis, the pins are pushed through the husk into the kernels, and 5 ml of inoculum (2×10^5 conidia/ml) are injected under the husk. A large number of kernels are damaged using this technique. Ear rot ratings of ears inoculated with the pinboard are highest when ears are inoculated 14 to 20 days after midsilk (Campbell and White, 1994). The pinboard inoculator was used to evaluate corn inbreds in Illinois and Mississippi (Olanya et al., 1997). The mean level of aflatoxin contamination in Illinois and Mississippi was 363 and 2,844 ppb, respectively. The pinboard inoculator severely wounds ears and may be impractical to use in the hot, humid South. However, it does provide a reliable method to evaluate corn genotypes in the temperate conditions of the Midwest (Campbell and White, 1995a,b; Naidoo et al., 2002; Walker and White, 2001).

The side-needle technique has been used for 16 years in Mississippi to evaluate corn germplasm for resistance to *A. flavus* (Zummo and Scott, 1989). This technique is a reliable method for inoculating corn ears in the field with minimal damage to developing kernels (Windham and Williams, 1998). The side-needle technique utilizes an Idico tree-marking gun fitted with a 14-gauge needle. Inoculations made with the side-needle technique are most effective 6 days after midsilk as opposed to 12 or 18 days after midsilk (Scott and Zummo, 1994). Multiple inoculations have not shown to increase the amount of kernel infection or aflatoxin contamination (Scott and Zummo, 1994). To inoculate ears using this technique, the needle is inserted under the husks on the upper 1/3 of the ear and 3.4 ml of a spore suspension (9×10^7 conidia/ml) is injected over the kernels. When

compared with ears inoculated with the pinbar and silk channel techniques, ears inoculated with the side-needle technique had similar levels of *A. flavus* kernel infection (Zummo and Scott, 1989). An advantage of the side-needle technique to the pinbar and silk channel techniques is the speed and ease of making the inoculations. The side-needle technique has recently been used to identify corn inbreds and advanced breeding lines with resistance to aflatoxin contamination (Williams and Windham, 2001; Windham and Williams, 2002). Although labor intensive, this technique has proven superior to other wounding and non-wounding inoculation techniques in Mississippi field evaluations.

The toothpick-under-husk technique (TUH) and the punch drill/pipe cleaner (PDPC) technique have also been used to evaluate resistance of corn genotypes (Wicklow et al., 1994; Zhang et al., 1998). Inoculum for the TUH technique is increased on toothpicks placed on a growth medium and then inoculated with *A. flavus*. Developing ears are inoculated by making an incision in the husks in the middle of the ear and inserting a toothpick. In field studies in Louisiana, the TUH technique produced higher levels of aflatoxin contamination and had less variability than 3 non-wounding inoculation techniques (Zhang et al., 1998). However, in a Mississippi study, ears inoculated with *A. flavus* using the TUH technique had lower levels of kernel infection than in ears inoculated with the pinbar or side-needle technique (Zummo and Scott, 1989). The PDPC technique has been used in hybrid evaluations at Weslaco, TX (Wicklow et al., 1994). Pipe cleaners inoculated with *A. flavus* were placed in holes drilled into the cob of developing ears. At harvest, non-wounded kernels were harvested from around the drilled holes and evaluated for aflatoxin contamination to determine resistance of Corn Belt hybrids.

Insect vectors have also been used to inoculate developing ears with *A. flavus* (Barry et al., 1985; Windham et al., 1999). In a study in Mississippi, a hand-operated dispenser previously developed to infest plants with lepidopterans was used to apply corn cob grits containing *A. flavus* spores onto corn silks inside a shoot bag (Windham et al., 1999). This application was followed 24 hours later by an application of southwestern corn borer neonate larvae. Inoculating the fungus and infesting silks with the insect dispenser worked well in producing high levels of aflatoxin contamination. This inoculation technique may also be useful in studying *A. flavus* interactions with other ear feeding insects.

Inoculum

The *A. flavus* strain and the conidial concentration of inoculum are both critical in providing adequate kernel infection in field evaluations.

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A. flavus isolates vary in their ability to infect corn kernels on developing ears in the field (Zummo and Scott, 1994). The NRRL 3357 isolate of *A. flavus* infected a higher percentage of kernels than an *A. parasiticus* isolate and other *A. flavus* isolates (Zummo and Scott, 1994). This isolate is a reliable producer of aflatoxin under field conditions and has been used in field studies for over 25 years at different locations (King and Scott, 1982; Widstrom et al., 1982; Windham and Williams, 2002; Zhang et al., 1998; Zummo and Scott, 1989). New NRRL 3357 cultures should be started every year prior to the growing season from freeze dried mycelial plugs which can be obtained from the USDA, ARS, Northern Regional Research Laboratory, Peoria, Illinois. To insure adequate infection of corn genotypes in the Midwest, mixtures of toxigenic strains of *A. flavus* have been used in field evaluations (Campbell and White, 1994; Walker and White, 2001). The amount of kernel infection of corn genotypes in field evaluations is dependent on the conidial concentrations of the inoculum. Ears inoculated with wounding and non-wounding techniques had highest levels of kernel infection when a conidial concentration of 10^6 was used compared to conidial concentrations of 10^4 and 10^5 (Zummo and Scott, 1989). *A. flavus* inoculum can be increased on a V8 vegetable juice medium or on Czapek solution agar amended with NaCL (Dorner et al., 1999; Zummo, 1991). Toxigenic strains of *A. flavus* should not be repeatedly transferred in culture in the laboratory. Serial transfers often result in the degeneration of *A. flavus* cultures and the loss of aflatoxin production (Horn and Dorner, 2001). Large amounts of *A. flavus* inoculum can be produced on sterilized wheat or on sterilized corn cob grits (Grit-O-Cobs, Maumee, OH) (Dorner et al., 1998; Windham and Williams, 1999). Conidial concentrations of spore suspensions can be quantified using a turbidity meter or a hemacytometer. Tween 20 or a spreader sticker (Hi-Yield Chemical Co., Bonham, Texas) should be added to spore suspensions to reduce surface tension and disperse the conidia.

Color mutants of *A. flavus* (white, tan) and *A. parasiticus* (reddish-brown) have been used in field studies to evaluate corn for aflatoxin resistance (Wilson et al., 1986; Zummo, 1991). These isolates are easily recognizable from wild-type isolates which are predominantly yellow-green or olive-green. *Aspergillus* species have been identified that produce norsolorinic acid (NOR) which is a visible orange intermediate of aflatoxin (Keller et al., 1994). An *A. parasiticus* isolate that produces NOR has been used in field evaluations to identify resistant corn genotypes (D. M. Wilson, pers. comm.). Ears inoculated with this NOR mutant were visually screened for fungal infection by counting kernels that had a reddening of the aleurone layer. The NOR mutant was used to identify highly susceptible genotypes in the initial stages of mass screenings in an effort to reduce

aflatoxin analyses and expenses. Transformants of *A. flavus* containing the green fluorescent protein (GFP) gene from the jellyfish *Aequorea victoria* have been developed and may be useful in future studies identifying resistant corn genotypes (Du et al., 1999).

Environmental Effects

Aflatoxin production in developing ears of corn naturally infected with *A. flavus* is influenced by a number of environmental conditions (Widstrom, 1996). Higher than normal ambient temperatures and drought conditions are commonly associated with aflatoxin outbreaks. Aflatoxin production in ears artificially inoculated with *A. flavus* can also be influenced by environmental conditions and huge fluctuations of aflatoxin contamination from year to year may result (Bock and Cotty, 1999; Olanya et al., 1997). It is imperative to choose an inoculation technique that will allow researchers to distinguish between resistant and susceptible genotypes consistently from year to year regardless of the environmental conditions. Wounding inoculation techniques are generally less susceptible to environmental influences than techniques that do not wound kernels. Ears inoculated by spraying conidia on silks may have low levels of *A. flavus* infection and little aflatoxin contamination when growing conditions do not favor fungal development. Also, inoculum deposited on the exterior surface of the ear may be exposed to unfavorable conditions such as high temperatures during mid day. Spray inoculations made in late afternoon have been successful in establishing the fungus in developing kernels (Cardwell et al., 2000).

EVALUATION OF AFLATOXIN RESISTANCE IN THE LABORATORY

Laboratory evaluations for detecting aflatoxin resistance in corn genotypes have been developed (Adams et al., 1984; Brown et al., 1995, 2001; Wallin, 1986). The advantages of using laboratory evaluations are being able to screen material year round, less expense compared to field evaluations, and fewer kernels are required for evaluations. A kernel-screening assay (KSA) has been used to quantify aflatoxin contamination and fungal growth in mature kernels (Brown et al., 1995, 2001). Kernels evaluted in KSA are surfaced sterilized, dipped into an *A. flavus* spore suspension, and incubated in 100% relative humidity at 31°C for 7 days. Aflatoxin contamination and fungal infection of kernels can then be quantified. An *A. flavus B*-glucuronidase (GUS) transformant was used in

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KSA studies to evaluate corn inbreds for resistance to fungal infection (Brown et al., 1995). KSA has also been used to confirm resistance identified in field studies and to identify potential new sources of resistance (Brown et al., 1995, 2001). This technique has also been used with wounding and non-wounding inoculation techniques to study resistance mechanisms (Brown et al., 1997, 1999).

OUTLOOK

Although tremendous progress has been made in developing inoculation techniques that can be used to identify aflatoxin resistant corn genotypes, an inoculation technique that could be used at multiple locations regardless of the environmental conditions is still needed. Inoculation techniques that require a minimum amount of labor and produce uniform levels of infection would be useful to commercial seed companies in the development of resistant hybrids. Research on factors that affect *A. flavus* infection and aflatoxin contamination under field conditions needs to continue in order to develop efficient evaluation procedures. Continued improvement of laboratory techniques for evaluating corn genotypes is also needed. The use of *A. flavus* transformants containing GUS and GFP reporter genes may lead to improved inoculation and evaluation techniques.

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